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Published in:
Immunity

DOI:
10.1016/j.immuni.2006.10.019

Publication date:
2007

Immunity
Article

Full Activation of the T Cell Receptor Requires Both Clustering and Conformational Changes at CD3

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DOI 10.1016/j.immuni.2006.10.019

SUMMARY

T cell receptor (TCR-CD3) triggering involves both receptor clustering and conformational changes at the cytoplasmic tails of the CD3 subunits. The mechanism by which TCRαβ ligand binding confers conformational changes to CD3 is unknown. By using well-defined ligands, we showed that induction of the conformational change requires both multivalent engagement and the mobility restriction of the TCR-CD3 imposed by the plasma membrane. The conformational change is elicited by cooperative rearrangements of two TCR-CD3 complexes and does not require accompanying changes in the structure of the TCRαβ ectodomains. This conformational change at CD3 reverts upon ligand dissociation and is required for T cell activation. Thus, our permissive geometry model provides a molecular mechanism that rationalizes how the information of ligand binding to TCRαβ is transmitted to the CD3 subunits and to the intracellular signaling machinery.

INTRODUCTION

Ligand binding and signal transmission functions of the T cell receptor (TCR-CD3) complex are located on different subunits. The TCR-CD3 is composed of the ligand-binding TCRαβ (or TCRγδ) heterodimer and the signal-transducing dimers of CD3εγ, CD3εδ, and ζζ. The variable immunoglobulin (Ig) domains of TCRα and TCRβ form the binding surface for its ligand, the major histocompatibility complex-peptide (MHCp). The TCRα and TCRβ constant Ig and transmembrane regions couple TCRαβ to the CD3 dimers (Call et al., 2002). The CD3 subunits contain an extracellular Ig domain, a transmembrane region, and a cytoplasmic tail including several signal-transduction motifs (Alarcon et al., 2003; Malissen, 2003). The stoichiometry of the TCR-CD3 complex is controversial, and several distinct stoichiometries might coexist on the cell surface (Schamel et al., 2005).

A central issue in T cell activation is to understand how the information of MHCp binding to TCRαβ is transmitted into the cell via the CD3-signaling units. Two main models have been put forward, involving TCR-CD3 clustering and conformational changes (Alarcon et al., 2003; Choudhuri et al., 2005; Cochran et al., 2001; Germain, 2001; Malissen, 2003; Sigalov, 2005). One model stipulates that TCR-CD3 clustering by multimeric MHCp brings individual TCR-CD3 complexes into close proximity, thereby enabling transphosphorylation of the receptors by associated tyrosine kinases. In support of this view, soluble monomeric MHCp, unlike dimeric or oligomeric MHCp, is unable to elicit TCR-CD3 activation (Abastado et al., 1995; Boniface et al., 1998; Cochran et al., 2000; Stone and Stern, 2006). Similarly, T cells can be stimulated by intact anti-CD3 or anti-TCRαβ, but not by the corresponding Fab fragments (Kaye and Janeway, 1984).

Alternatively, conformational changes in the TCR-CD3 complex upon antibody binding have been proposed to explain T cell-signaling studies because differences in receptor clustering or in antibody affinities were insufficient to explain distinct activation potentials of TCR-CD3 antibodies (Janeway, 1995). However, with one exception (Kjer-Nielsen et al., 2003), crystallographic studies argue against large conformational changes within the TCRαβ Ig ectodomains. Structures from MHCp-bound and free soluble TCRαβ revealed ligand-induced structural changes in the complementarity-determining regions of the variable Ig domains of TCRαβ that were not transmitted to TCRαβ-constant regions (Bankovich and Garcia, 2003; Ding et al., 1999; Reiser et al., 2002; Rudolph et al., 2006). It is therefore difficult to envisage how ligand-induced conformational changes could be transmitted from the TCRαβ heterodimer to the CD3 tails.

Despite these conceptual problems, the TCR-CD3 complex undergoes a ligand-induced conformational change that allows a conserved proline-rich sequence (PRS) in the cytoplasmic tail of CD3ε to bind to the first SH3 (src homology 3) domain of Nck (SH3.1(Nck)), a ubiquitously expressed adaptor protein (Gil et al., 2002). This structural change can be induced by anti-CD3 and anti-TCRαβ as well as by MHCp (Gil et al., 2002, 2005; Risueno et al., 2005). To date, it is unclear whether Nck recruitment to TCR-CD3 is a crucial step in T cell activation (Gil et al.,

2002; Szymczak et al., 2005) and whether conformational changes are required for TCR-CD3 triggering. However, the conformational change is probably a more global event that also affects the other signaling subunits of the TCR-CD3 and additional signaling molecules besides Nck. Indeed, the cytoplasmic tail of the ζ chain might convert from a lipid-bound helical structure to an unfolded structure upon TCR-CD3 triggering (Aivazian and Stern, 2000).

By using TCR-CD3 ligands of defined valencies and geometries, we demonstrated that induction of the conformational change required both multivalent engagement and the mobility restriction imposed by the membrane. So far, TCR-CD3 clustering and the conformational change were discussed as two independent mechanisms to activate T cells and were not integrated into a unique model of TCR-CD3 triggering. We showed that TCR-CD3 clustering was a prerequisite for inducing the conformational change, and it further required the reorientation of two TCRαβ heterodimers with respect to each other. This reorientation resulted in rearrangements within the TCR-CD3 complex promoting the conformational change. We also demonstrated that the conformational change of CD3 was necessary, but not sufficient, for optimal T cell activation and was reversible upon dissociation of TCR-CD3-ligand complexes.

RESULTS

Multivalent Engagement of TCRαβ Is Required to Induce the Conformational Change

The conformational change leading to exposure of the PRS of CD3ε is induced upon binding of both bivalent and monovalent antibodies to CD3ε (Gil et al., 2002). To determine whether stimulation with monomeric and multimeric MHCp changes the conformation of CD3ε, we took advantage of T cells expressing the T1 TCR. This TCR recognizes the PbCS252-260 peptide (SYIPSAEKI) containing a photoreactive 4-azidobenzoic acid on K259 (pepABA) (Gregoire et al., 1996) in the context of K^d. After binding to the T1 TCR, photoactivation of ABA results in covalent crosslinking of TCRαβ with MHCp (Doucey et al., 2003; Gregoire et al., 1996), thereby ''freezing'' the interaction between MHCp and TCR-CD3.

Monomeric and tetrameric versions of the K^dpepABA were used to stimulate CD8-negative T1 hybridoma cells. Both could bind to the T1 TCR, as seen by flow cytometry (data not shown). After photocrosslinking, the conformational change in CD3ε was detected by the pull-down assay with the immobilized SH3.1(Nck) domain. The MHCp tetramer and CD3 antibody were equally potent in inducing the conformational change (Figure 1A, lanes 3 and 4). In contrast, monomeric MHCp was not able to induce any conformational change (lane 2), although it bound to the T1 TCR as shown by the presence of β2-microglobulin in the anti-CD3 immunoprecipitates (lowest panel). A comparison of MHCp dimer and tetramer indicated that both have the same capacity to induce the conformational change (Figure 1B). In contrast, not all TCR-CD3 antibodies had equal capability to induce the conformational change in CD3ε (see Figure S1 in the Supplemental Data available online). Likewise, complete TCRαβ antibodies but not their monovalent Fab fragments induced the conformational change (Figure S1). These data showed that monovalent MHCp binding to TCRαβ did not induce the conformational change in TCR-CD3 and that bivalent or multivalent MHCp engagement was required. Hence, induction of the conformational change in CD3ε via TCRαβ requires TCR-CD3 clustering.

In the absence of photocrosslinking, the K^dpepABA is not covalently fixed to the T1 TCR (Gregoire et al., 1996). To test whether the conformational change is dependent on continuous engagement, T1 hybridomas were incubated with MHCp tetramers or CD3 antibodies and subsequently UV irradiated or left unexposed. The CD3ε conformational change was preserved only when the MHCp-TCR-CD3 interaction was covalently fixed. When MHCp was not photocrosslinked to TCRαβ, the conformational change was not detected (Figure 1C, lane 2). Nevertheless, in both cases MHCp was bound to TCR-CD3 (Figure 1D). Upon detergent lysis, MHCp rapidly dissociates from TCRαβ (Arcaro et al., 2001), probably because of the low MHCp-TCRαβ affinity. Indeed, the MHCp tetramer was not detected in an anti-TCR-CD3 immunoprecipitation when UV irradiation was omitted (Figure 1C, lowest panel), whereas the induction of the conformational change by CD3 antibodies was independent of UV irradiation. The experiment was repeated with a T1 CTL clone (Doucey et al., 2003) with similar results (Figure S2). Thus, disruption of MHCp binding most likely results in reversion of the conformational change.

Next, we tested whether MHCp expressed by antigen-presenting cells (APCs) can induce the conformational change. K^d-expressing APCs were loaded with pepABA and used to stimulate CD8-negative (Figure 1E) and CD8-positive (Figure 1F) T1 hybridoma cells. Indeed, stimulation with APCs induces the conformational change, and this induction is independent of CD8 expression. Thus, induction of the conformational change via TCRαβ by MHCp requires multivalent engagement in absence of any additional interaction.

Close Proximity of Two TCR-CD3 Is Necessary to Induce the Conformational Change

To exclude that ligand binding was directly altering the conformation of the TCRαβ heterodimer, we designed a system in which the ligand did not directly bind to TCRαβ, but only to an appended immunoglobulin single-chain (sc) construct. The V_H and V_L regions of an anti-hapten (nitro-iodo-phenol, NIP)-specific antibody were made as one single-chain molecule and fused to the N terminus of a mature TCRβ chain through a flexible linker (Figure 2A). The resulting NIP-specific single-chain TCRβ protein (scTCRβ) was stably expressed in a TCRβ-deficient Jurkat mutant (31-13), yielding the cell line 31-13.scTCRβ. The scTCRβ was expressed within the TCR-CD3 complex on the cell surface (Figure S3). Because hapten binding to antibodies does not lead to a change in the structure

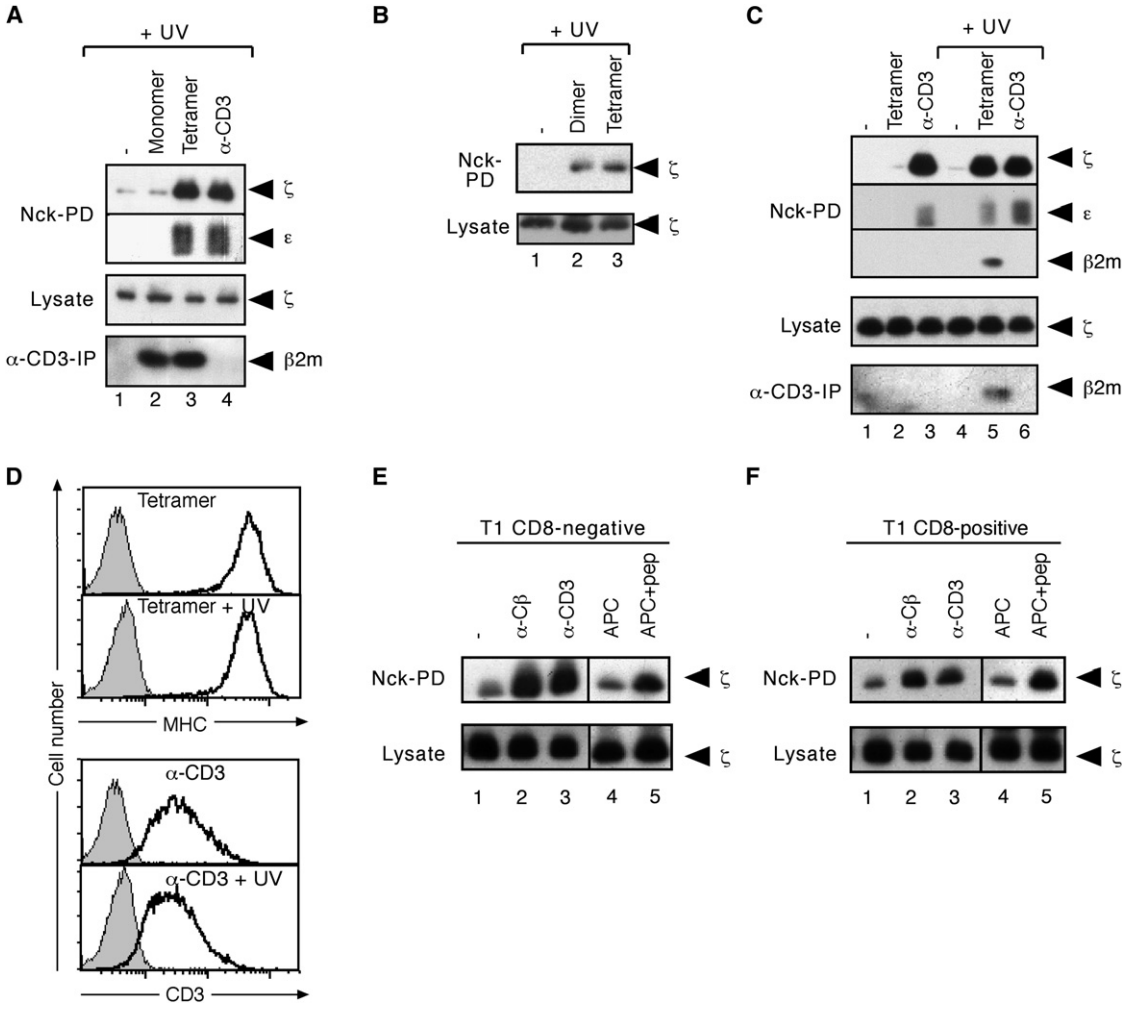

Figure 1. Only Multimerized MHCp Induce the Conformational Change in CD3

(A) T1 hybridoma T cells were incubated with 500 nM of K^dpepABA monomer, 5 nM of K^dpepABA tetramer, or 5 μg/ml of CD3 antibody (145-2C11) corresponding to maximal TCR-CD3 binding. Cells were UV irradiated to covalently crosslink the MHCp to the TCR-CD3. Upon lysis, the Nck pull-down assay was performed and TCR-CD3 detected by anti-ζ and anti-CD3ε immunoblotting (top). As control, an aliquot of each lysate was subjected to anti-ζ immunoblotting to confirm equal amount of TCR-CD3 (middle). Alternatively, an anti-CD3 immunoprecipitation was done and presence of MHC detected with a β2-microglobulin antibody (bottom).

(B) T1 hybridoma cells were incubated with 50 nM K^dpepABA dimer or 5 nM tetramer that resulted in maximal TCR-CD3 binding and subjected to UV irradiation. Nck pull-down was performed as in (A).

(C) T1 hybridoma was incubated with 5 nM K^dpepABA tetramer or 5 μg/ml anti-CD3. Cells were UV irradiated or left untreated and processed as in (A).

(D) Aliquots of the samples from (C) were used to confirm stimuli binding. The tetramer already included streptavidin-PE (middle), and bound anti-CD3 was detected with anti-IgG-PE (bottom). Untreated cells are shown in gray.

(E) T1 hybridoma T cells lacking CD8 expression were stimulated with pepABA-pulsed A20 cells 10 min at 37°C. Upon UV irradiation and paraformaldehyde fixation, cells were lysed and processed as above. As a control, T1 cells were stimulated with the indicated antibodies or left untreated.

(F) T1 hybridoma T cells expressing CD8 were stimulated with pepABA-pulsed A20 cells, and induction of the conformational change was probed as in (E).

The results are representative of at least three independent experiments.

outside the hapten-binding pockets (Wedemayer et al., 1997) and because the sc molecule was fused to the TCR-CD3 via a flexible linker, it is highly unlikely that NIP binding could transmit a structural change to TCRαβ.

To test whether monovalent or multivalent engagement of the scTCRβ could induce the conformational change, we generated a series of peptides in which the number of NIP molecules coupled to each peptide was increased from one to four (Figure 2B). For comparison, NIP-coupled bovine serum albumin (BSA) was used in which one BSA molecule was conjugated on average to 15 NIP molecules (NIP15-BSA). Stimulations were performed maintaining equimolar amounts of the NIP moiety (Figure S3). The monovalent NIP1 peptide did not induce the conformational change (Figure 2C, lane 2), nor did monomeric free hapten (data not shown). However, as the number

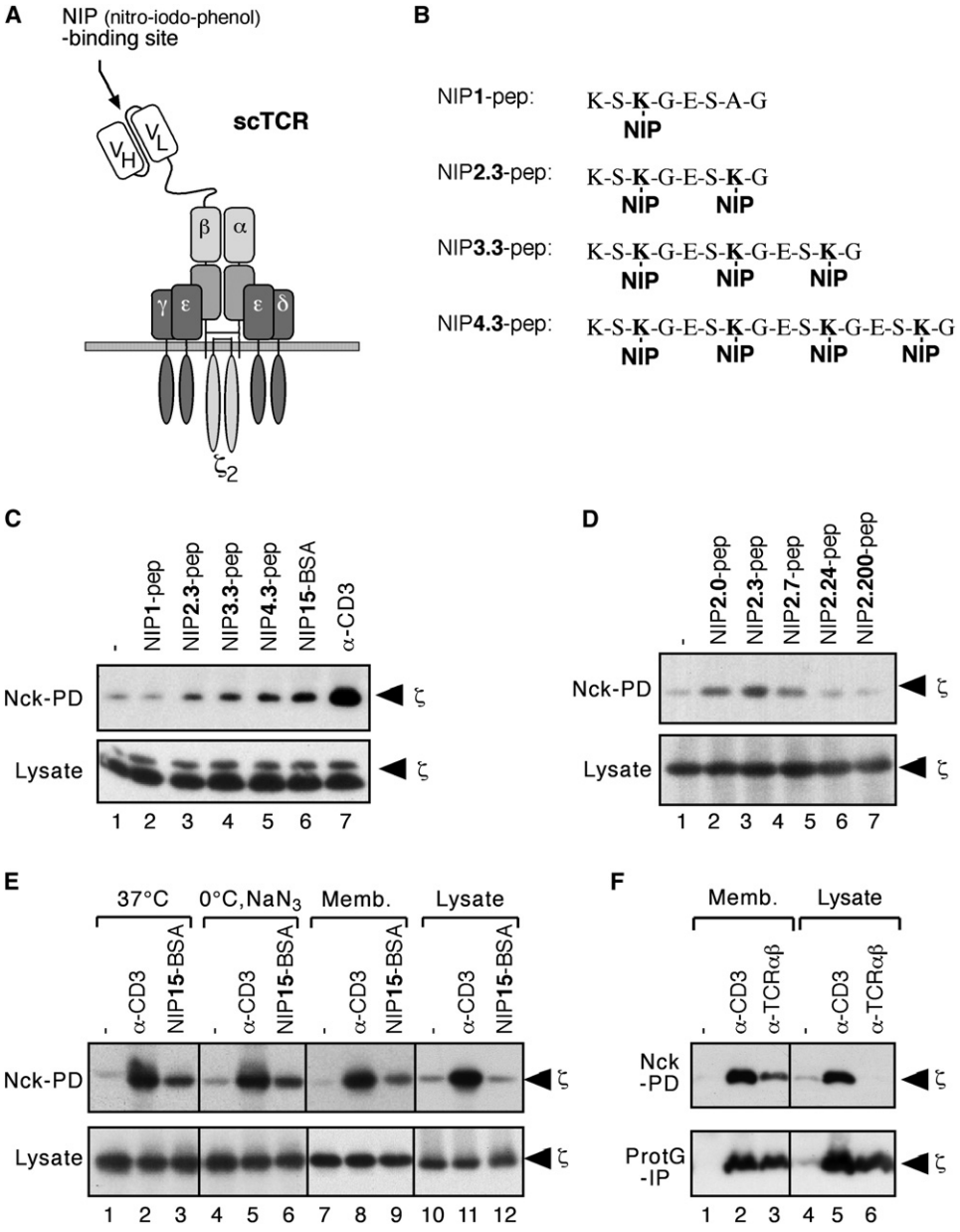

Figure 2. Simultaneous Engagement of Several TCRαβ Is Sufficient to Induce the Conformational Change

(A) scTCRβ is composed of the NIP binding variable immunoglobulin domains of a NIP antibody connected by a flexible linker of 8 amino acids to the N terminus of wild-type TCRβ.

(B) The sequence of the peptides that contain the NIP-conjugated lysine are shown. The one letter code for amino acids is used. The first number in the name of the peptide indicates the number of NIP-conjugated lysines and the second number indicates the number of amino acids between two adjacent NIP-conjugated lysines.

(C) 31-13.scTCRβ cells were stimulated with the indicated NIP-coupled peptides (lanes 2–5), NIP15-BSA, anti-CD3ε (UCHT1), or left untreated. Concentrations of the NIP-coupled reagents were chosen in order that the number of NIP molecules per stimulation was constant. After lysis, the Nck pull-down assay was performed as in Figure 1.

(D) 31-13.scTCRβ cells were either stimulated with equal molarities of the indicated peptides or left untreated. Upon lysis, the Nck pull-down was assayed as in (C).

(E) 31-13.scTCRβ cells were stimulated at 37°C or on ice in the presence of azide. Alternatively, a membrane fraction of 31-13.scTCRβ cells was incubated with the stimuli on ice, or stimuli were added to the detergent lysates. The stimuli were NIP15-BSA and CD3 antibody. The Nck pull-down assay was done as in (C).

(F) A membrane fraction of SRD10 cells was incubated on ice with anti-CD3 (145-2C11) or anti-TCRαβ (3D3). Alternatively, the antibodies were added to the lysates. After lysis of the membranes, the Nck pull-down assay was done as above. As control for antibody binding, protein G-coupled sepharose was incubated with the corresponding lysates and the presence of the antibody-TCR-CD3 complex was assayed (bottom).

The results are representative of at least three independent experiments.

of NIP molecules per peptide increased, the PRS of CD3ε became increasingly accessible (Figure 2C). Thus, simultaneous engagement of several TCR-CD3 complexes, but not a change in the TCRαβ structure, is necessary and sufficient to induce the conformational change.

We then examined how the distance between two NIP ligands influences the ability to induce the conformational change. To this end, we synthesized peptides with varying numbers of amino acids between two NIP-conjugated lysines. The NIP2.0 peptide contained no spacer between the two NIP lysines, the NIP2.3 peptide has a three amino acid spacer, etc. (Figure S3). An increase in the distance between the two haptens within a peptide resulted in decreased induction of the conformational change (Figure 2D). With a spacer of 24 amino acids or more, the peptide ligands did not induce the conformational change. We excluded the possibility that the bivalent NIP peptides bound to two TCR-CD3s on two different T cells (Figure S4). Hence, our data indicate that two TCRαβ have to be brought into close proximity and/or in a specific orientation in order to transduce the conformational change.

The Mobility Restriction Imposed by an Intact Membrane Is Necessary to Induce the Conformational Change via TCRαβ

The above results argue that two or more TCR-CD3s have to be contacted in order to change their conformation. This does not depend on metabolic processes because the PRS was also exposed when T cells were stimulated in the presence of azide at 0°C (Figure 2E, lanes 4–6). The conformational change also occurred on isolated membrane patches prepared in the absence of detergent, indicating that cytosolic proteins were not involved in the process (lanes 7–9). However, when detergent-solubilized TCR-CD3s were incubated with NIP15-BSA, the conformational change was not induced (lane 12). We could not demonstrate that NIP15-BSA bound to TCR-CD3 in the lysate. Therefore, we repeated the experiment with SRD10 cells stimulated with an idiotypic antibody. The TCRαβ antibody induced the conformational change on membrane preparations but not in detergent lysates (Figure 2F, lanes 3 and 6). Here we demonstrated that the antibody was bound to the TCR-CD3 in the lysates by immunoprecipitation with ProteinG-Sepharose beads (Figure 2F, bottom). In both experiments, anti-CD3ε induced the conformational change in detergent lysates by binding directly to CD3ε (Figures 2E and 2F). These results indicate that induction of the conformational change via TCRαβ clustering requires an intact membrane.

The Conformational Change in CD3 Reverts upon TCRαβ-Ligand Dissociation

As we showed in Figure 1, covalent fixation of the T1 TCR-CD3 to its cognate MHCp was necessary to detect the conformational change in CD3ε. This could be explained if the conformational change was reverted upon ligand detachment during cell lysis. To directly test this hypothesis, we used our scTCR system, in which the ligand can be removed under controlled conditions (Figure 3A). Binding of

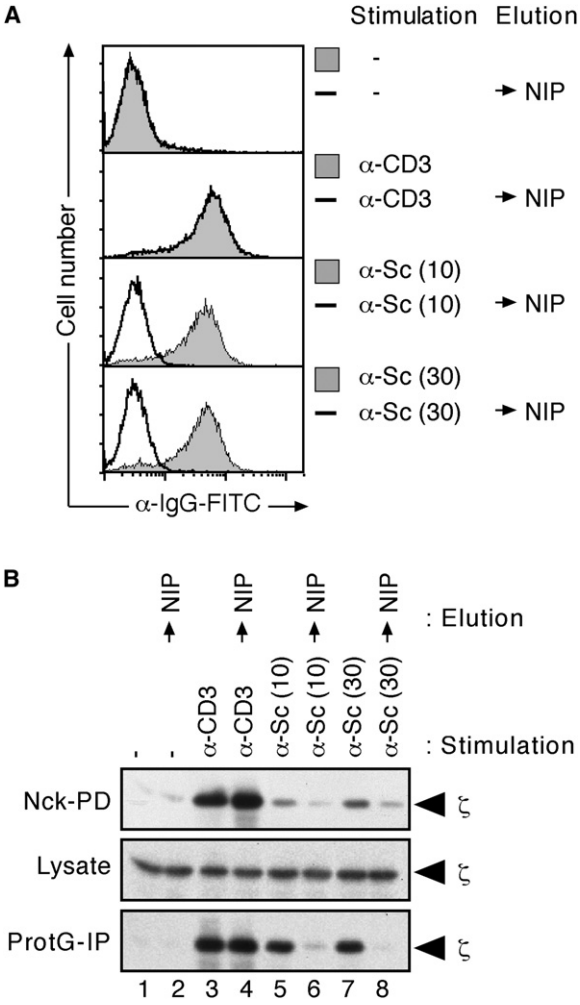

Figure 3. The Conformational Change Is Reversible

(A) 31-13.scTCRβ cells were left unstimulated and either subsequently treated with 1 mM free monomeric NIP (top, solid line) or left untreated (gray graph). Alternatively, the cells were stimulated with anti-CD3ε and either treated with free NIP (second panel, solid line) or left untreated (gray graph). Lastly, sc antibody (10 μg/ml, third panel or 30 μg/ml, last panel) was used, in combination with or without subsequent elution with NIP (solid line or gray graph, respectively). Cells were stained with anti-IgG and analyzed by flow cytometry.

(B) Anti-CD3ε (10 μg/ml) and anti-sc (10 μg/ml or 30 μg/ml)-stimulated 31-13.scTCRβ cells were left untreated (lanes 3, 5, 7) or treated with 1 mM NIP (lanes 4, 6, 8). The Nck pull-down assay was performed as in Figure 1. Protein G-coupled sepharose was incubated with an aliquot of the corresponding lysates and the presence of antibody-TCR-CD3 complexes was assayed (bottom). The samples were from the same experiment as in (A).

The results are representative of at least four independent experiments.

the sc antibody Ac146 to the hapten-recognition site of the sc domain was inhibited by incubation with the free hapten NIP (Reth et al., 1979). When 31-13.scTCRβ cells were incubated with the sc antibody, the conformational change was detected (Figure 3B, lanes 5 and 7). However, the conformational change was reverted when the sc antibody was removed by adding an excess of monovalent NIP to the living cells (lanes 6 and 8). Note that similar to

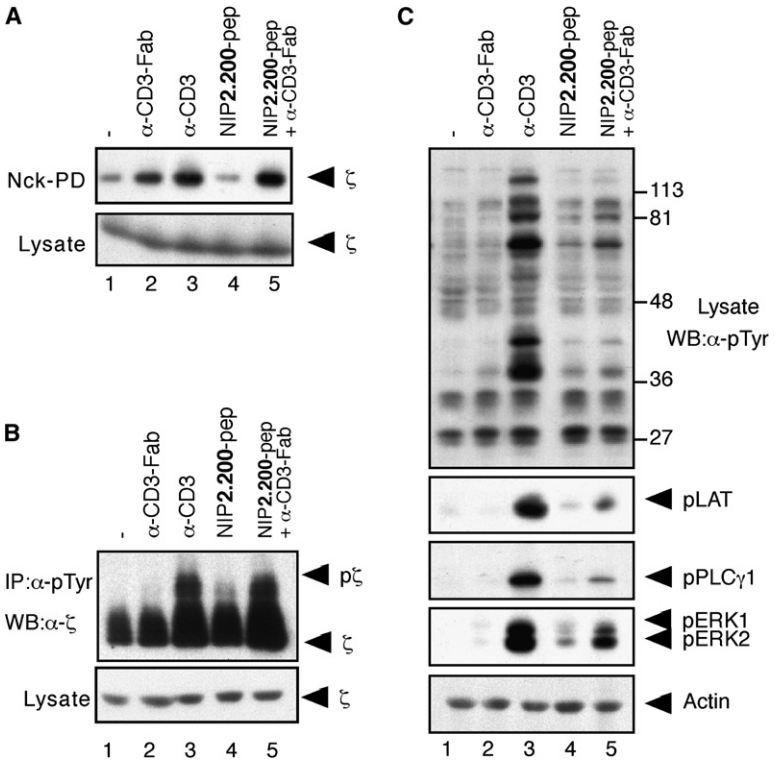

Figure 4. The Conformational Change Is Required for TCR-CD3 Signaling

(A) 31-13.scTCRβ cells were incubated with the indicated stimuli 3 min at 37°C. The stimuli were: 5 μg/ml of the anti-CD3-Fab fragment or the CD3 antibody (OKT3) as well as 2.8×10^4 NIP molecules/ml of the NIP2.200 peptide. Upon lysis, the Nck pull-down assay was performed as before.

(B) Stimulation of 31-13.scTCRβ cells was done as in (A). After lysis, an anti-phosphotyrosine immunoprecipitation was carried out and the purified proteins were detected by anti-ζ immunoblot (top). Anti-ζ immunoblotting of an aliquot of the lysate verifies equal loading.

(C) 31-13.scTCRβ cells were stimulated as in (A). The lysate was separated by SDS-PAGE and the indicated proteins were detected by immunoblotting with anti-phospho-tyrosine (4G10) or anti-phospho-specific antibodies as indicated. Anti-actin immunoblotting serves as control.

The results are representative of at least three independent experiments.

the bivalent NIP2 peptides (Figure 2C), the anti-sc antibody is a weak inducer of the conformational change. As a control, incubation of 31-13.scTCRβ cells with monomeric NIP had no effect on the conformational change induced by anti-CD3 (Figure 3B, lanes 3 and 4). Taken together, these results as well as those observed for the MHCp and the T1 TCR-CD3 (Figure 1) show that the TCR-CD3 reverts to its basal conformation when the stimulus dissociates.

The Conformational Change Is Necessary for T Cell Activation

Whereas overexpression of the CD3ε binding SH3 domain of Nck (SH3.1) inhibited T cell activation (Gil et al., 2002), mutation of the PRS in CD3ε did not affect T cell development or antibody stimulation of T cells (Szymczak et al., 2005). Therefore, it is controversial whether Nck recruitment is important for T cell activation. We should stress that the Nck pull-down, and therefore the exposure of the PRS, is used in this study as a marker for the presence of the conformational change. However, because in addition to Nck the conformational change possibly has other effectors, we addressed whether lack of conformational change impairs T cell activation. 31-13.scTCRβ cells were stimulated with NIP-coupled peptides containing two NIP moieties separated by spacers of different length. Induction of the conformational change (Figure 2D) correlated with the ability of the NIP-modified peptides to stimulate Ca^{2+} influx and to upregulate the activation marker CD69 (Figure S5). Hence, poor inducers of the conformational change were also inefficient T cell activators.

To directly assess whether the conformational change is required for T cell activation, we used two different reagents. First was the NIP2.200 peptide, which does not induce the conformational change (Figure 2D) but simultaneously binds to two TCR-CD3s, and second was a Fab fragment of the CD3 antibody OKT3, which induces the conformational change (Figure 4A; Gil et al., 2002) but does not cluster the TCR-CD3. Simultaneous incubation with both reagents induced the conformational change (Figure 4A). When ζ phosphorylation was measured, we found that either stimulus alone was hardly active (Figure 4B), whereas both together induced strong phosphorylation, similar to that evoked by stimulation with bivalent anti-CD3 (Figure 4B). Thus, induction of the conformational change is required, but not sufficient, for optimal ζ phosphorylation.

Phosphorylation of TCR-CD3 leads to the recruitment of ZAP-70, which in turn phosphorylates several substrates including the adaptor proteins LAT and SLP-76. These proteins serve as docking sites to organize multiprotein complexes resulting in phosphorylation of phospholipase Cγ1, the MAP kinases ERK1 and ERK2, and activation of gene transcription factors such as NF-AT and NF-κB. To study the contribution of the conformational change to these events, we analyzed phosphorylation of several substrates with phospho-specific antibodies (Figure 4C). Only the simultaneous stimulation with anti-CD3-Fab fragments and NIP2.200 peptide caused substantial phosphorylation of LAT, PLCγ1, and ERK.

An important early event upon TCR-CD3 triggering is Ca^{2+} flux into the cytoplasm. Stimulation with

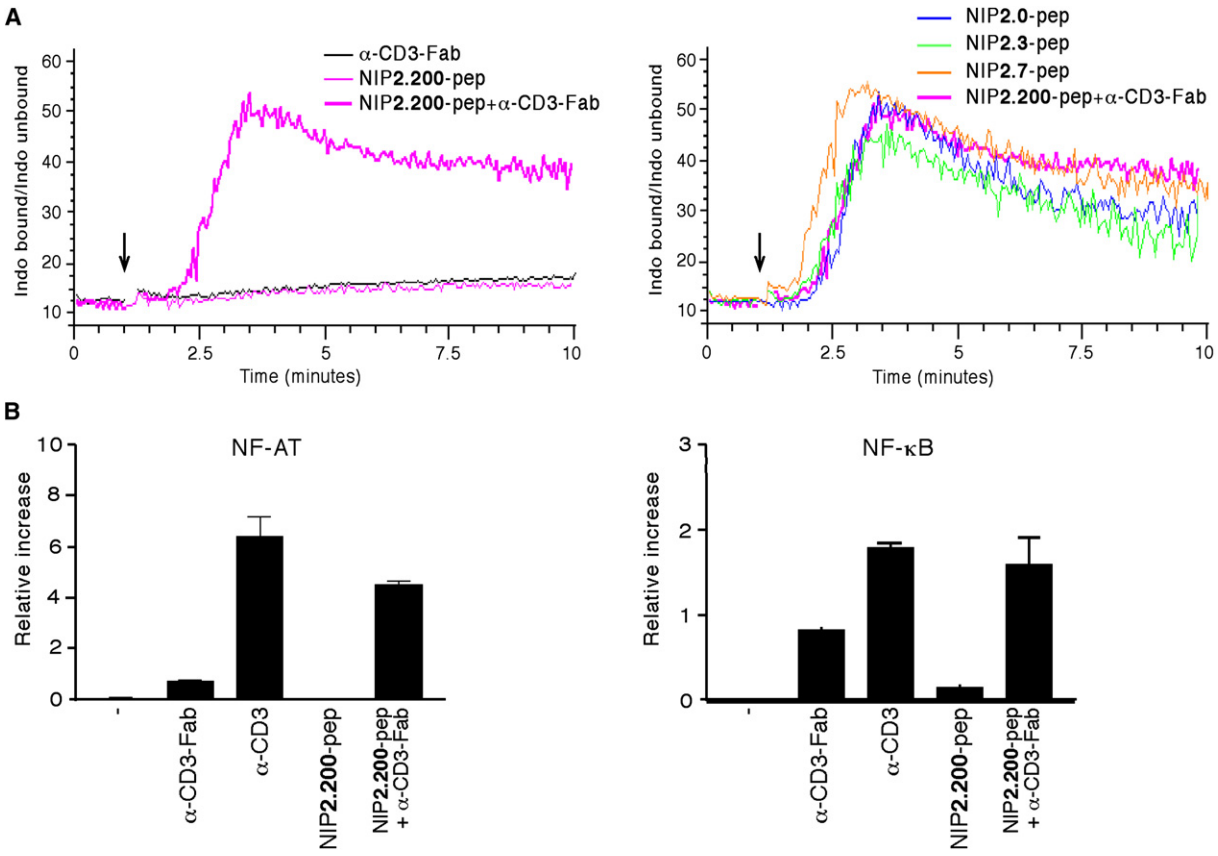

Figure 5. The Exposure of the PRS Is Crucial for T Cell Activation

(A) 31-13.scTCRβ cells were loaded with Indo-1 and stimulated with the indicated reagents (concentrations as in Figure 4) to induce Ca^{2+} responses. Equal molarities of the NIP-coupled peptides were used. The Indo-1 ratio was integrated over 10 min and measured by flow cytometry. The stimuli were added after 1 min (arrow).

(B) Upon transfection with an NF-AT (left) or an NF-kB (right) reporter plasmid, 31-13.scTCRβ cells were incubated 6 hr with the described stimuli and lysed, and luciferase activity was assayed relative to unstimulated cells. The mean of three independent, simultaneously performed stimulations is shown. Error bars indicate standard deviation.

The results are representative of at least five independent experiments.

anti-CD3-Fab or NIP2.200 peptide alone was not sufficient to induce a Ca^{2+} response. However, combination of both stimuli induced a strong Ca^{2+} flux indistinguishable from the one elicited by the other NIP2 peptides (Figure 5A). This indicates that it was the inability of the NIP2.200 peptide to induce the conformational change that accounted for its poor activity. These data also show that the distance of the two NIP moieties on the peptides did not play a substantial role in activation, as long as the conformational change is induced. The need for TCR-CD3 clustering was also demonstrated in this system, because a combination of the monovalent NIP1 peptide with the Fab fragment did not give any Ca^{2+} response, whereas clustering the Fab fragment with light chain antibodies did result in Ca^{2+} flux (Figure S5). In agreement with these results, optimal activation of NF-AT and NF-κB (Figure 5B) was detected only in the presence of both TCR-CD3 clustering and conformational change in the 31-13.scTCRβ cell line. Stimulation with the Fab fragment alone resulted in some activity, probably resulting from Fab multimerization by the tissue culture plate.

To confirm our data with primary T cells, we transiently transfected human peripheral blood mononuclear cells (PBMCs) with an expression vector encoding for the scTCRβ chain together with a GFP marker for transfected cells. Only stimulation with the anti-CD3-Fab and the NIP2.200 peptide resulted in a marked CD69 upregulation in the transfected, GFP-positive cells. As a control, no effect of the NIP2.200 peptide was seen in nontransfected, GFP-negative cells (Figure 6).

These results argue that it is possible to separate the conformational change from TCR-CD3 clustering by increasing the distance between binding groups in a bivalent ligand. With this approach we demonstrate that the conformational change in the TCR-CD3 complex is necessary for full T cell activation.

DISCUSSION

A key finding of the present study is that the relationship between the two models of TCR-CD3 triggering, namely

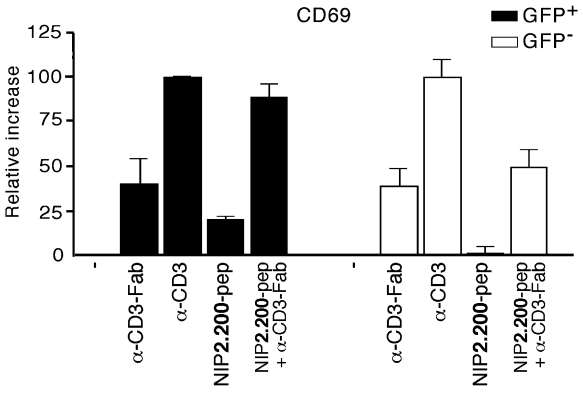

Figure 6. The Conformational Change Is Required for T Cell Activation in Human PBMCs

Human PBMCs were transiently cotransfected with vectors encoding for scTCRβ and GFP. The cells were stimulated (concentrations as in Figure 4) 24 hr or left untreated. After staining with a CD69 antibody, cells were analyzed by flow cytometry gating on GFP-positive (left) and GFP-negative (right) cells. The relative increase was calculated considering the percent of CD69-positive cells upon anti-CD3 stimulation as 100%. The mean of triplicates and the standard deviation are indicated. The results are representative of at least two independent experiments.

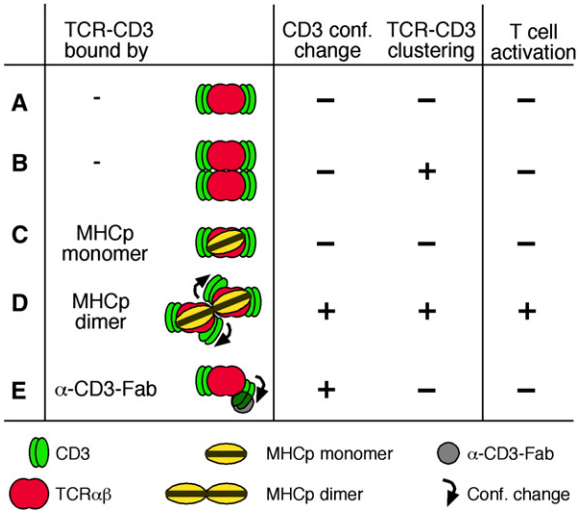

Figure 7. The Permissive Geometry Model of TCR-CD3 Triggering

(A) In absence of ligand binding, the TCR-CD3 complex is in a resting state.

(B) Clustering of two (or more) TCR-CD3 in a nonpermissive geometry that does not induce the conformational change is not sufficient for TCR-CD3 triggering. Inactive clustering is achieved by the NIP2.200 peptide. The TCR-CD3 might also be preclustered in the nonpermissive geometry before ligand binding (Schamel et al., 2005).

(C) Monovalent MHCp binding does not lead to a structural change of the TCRαβ heterodimer, thus not rearranging the cytoplasmic tails of CD3. Consequently, TCR-CD3 is not triggered.

(D) Bivalent (and multivalent) MHCp binding does not change the structure of one TCRαβ outside the direct contact region. Since two TCR-CD3 are engaged simultaneously, they have to adjust to the geometry of the preformed MHCp dimer. This results in a reorientation of two TCRαβ into a permissive geometry, thereby "pushing and squeezing" the extracellular and transmembrane regions of the CD3 and ζ subunits (arrows). The rearrangement is transmitted through the membrane and affects the conformation of the cytoplasmic regions of CD3.

(E) Fab fragments of CD3ε antibodies alter the conformation of CD3 by the monovalent direct binding event. The conformational change alone is not sufficient for TCR-CD3 triggering and T cell activation.

clustering and conformational change, is closer than anticipated because clustering is required to induce the conformational change (summarized in Figure 7). Remarkably, we found that monovalent engagement of TCRαβ by MHCp or anti-TCRαβ Fab fragments did not transmit a conformational change to the cytoplasmic region of CD3ε, whereas bivalent and multivalent engagement did. This implies that TCRαβ ligation per se is not sufficient to transmit structural alterations through the TCRαβ heterodimer to the CD3 subunits. Indeed, ligand binding generates induced-fit type changes only in the variable TCRαβ regions at the ligand-binding interface (Bankovich and Garcia, 2003; Reiser et al., 2002; Rudolph et al., 2006; Wu et al., 2002), but no alterations at the distal portions of the heterodimer, which are connected to the CD3 units. Note that unlike Fab fragments of antibodies against TCRαβ, Fab fragments of the CD3 antibodies OKT3 and UCHT1 induce the conformational change by binding directly to CD3ε (Figure S6).

To combine the rigidity of TCRαβ with the presence of conformational changes in CD3, a piston-like model, in which TCRαβ are displaced perpendicular to the membrane, and a rotational model, where TCRαβ rotate with respect to CD3, have been proposed (Choudhuri et al., 2005; Gil et al., 2005; Sun et al., 2001). These models imply that the ligand exerts a mechanical force on TCRαβ, which is possible only if a second fixed interaction is present. The coreceptors CD8 and CD4 could theoretically provide this interaction. We show that soluble bivalent MHCp or peptide-pulsed APCs induce the conformational change in the CD8-negative T1 hybridoma, indicating that CD8 is not involved in inducing the conformational change. Furthermore, anti-TCRαβ can induce the conformational change in the absence of any additional interaction. However, CD8 and CD4 can strengthen the MHCp-TCR-CD3 interaction, and therefore could aid in stabilizing the conformational change. Our data thus argue that the second fixed interaction is the second TCRαβ. Bivalent ligand binding could change the relative orientation of two TCRαβ dimers toward each other, enforcing cooperative interactions. After bi- or multivalent binding, the two TCRαβ might "pinch" the CD3 subunits so that the extracellular parts of CD3 are pushed away from their original positions. Because the short stalks connecting the extracellular domains and the transmembrane regions of CD3 appear to be rigid (Arnett et al., 2004; Sun et al., 2001), such displacement could be transmitted to the transmembrane domains, resulting in rearrangements of the cytoplasmic tails (Figure 7).

When TCRαβ were clustered in detergent lysates, the conformational change in CD3ε was not induced. This indicates that two TCRαβ need to be brought not only into close proximity (because increased distance led to

a reduced change), but also into a defined orientation within the constraints of the membrane. If the TCR-CD3 dimer does not have the right orientation, the conformational change is not induced. Thus, inducing proximity of two TCRαβ is necessary, but not sufficient, to generate the conformational change. Likewise, not all TCR-CD3 antibodies have the same capability to induce the conformational change, even when they bind to the same number of TCR-CD3s. Because these antibodies bind to distinct regions of TCR-CD3, they should lead to different geometries of the clustered complex. We therefore suggest that the exact geometry determines whether a conformational change takes place or not. A permissive geometry would lead to structural reorganization to expose the PRS of CD3ε, whereas a different inert geometry would not (permissive geometry model).

Further support for the permissive geometry model comes from our experiments with the scTCRβ chimera, in which the hapten binding domain (sc) is connected via a flexible linker to TCRβ. Engagement by a bivalent hapten or a bivalent anti-sc antibody probably brings two TCRαβ together in a random geometry. Therefore, only a few TCR-CD3 form the correctly oriented clustered αβ-αβ complex capable of communicating the conformational change to CD3. Increasing the valence of the haptenated peptides augments the probability of creating permissive geometries, which explains the increase in conformationally changed TCR-CD3s. Even NIP15-BSA was less efficient than anti-CD3 to induce the conformational change. In contrast, MHCp dimers, tetramers, and anti-CD3 were equally potent in inducing the conformational change, suggesting that because of conserved interactions between two MHCp molecules (Krishna et al., 1992; Schafer et al., 1995), the binding of dimeric MHCp already places all TCR-CD3s in the permissive geometry. Thus, TCR-CD3 dimers in the permissive geometry are necessary and sufficient to induce the conformational change of the TCR-CD3 complex.

In the present study, we address for the first time the importance of the ligand-induced conformational change in CD3, in contrast to previous reports that have studied only the role of the PRS of CD3ε (Szymczak et al., 2005) or the recruitment of Nck to this PRS (Gil et al., 2002). One of the consequences of the conformational change is the exposure of the PRS of CD3ε (Gil et al., 2002). By the use of a mutant TCR-CD3 complex lacking the PRS of CD3ε (Szymczak et al., 2005), only the importance of the PRS can be studied and not the role of the conformational change itself. To directly study the contribution of TCR-CD3 clustering and conformational changes to TCR activation, we used the Fab fragment of the CD3 antibody OKT3, which by direct binding induces the conformational change (Gil et al., 2002) but does not cluster TCR-CD3. In addition, the NIP2.200 peptide does not induce the conformational change, but does bind simultaneously to two TCR-CD3s. By combining the NIP2.200 peptide and anti-CD3 Fab, we show that both TCR-CD3 clustering and conformational changes are needed for optimal T cell activation.

The necessity of the conformational change for effective TCR-CD3 signaling in combination with the permissive geometry model might also explain the fact that all TCRαβ adopt a diagonal orientation on MHCp (Rudolph et al., 2006). Conserved MHCp-TCR-CD3 interactions that dictate this orientation are not apparent. We suggest that initially other orientations exist, but that the MHC self-peptide-TCR-CD3 interaction in the thymus selects those TCR-CD3s that bind to multivalent MHCp in the permissive geometry, i.e., the conformational change might be necessary for thymic selection to guarantee optimal T cell activation in the periphery. In addition, the diagonal orientation might ensure that MHCp-bound CD8 or CD4 will contact the TCR-CD3 complex at the correct position (Bankovich and Garcia, 2003; Buslepp et al., 2003; Garboczi et al., 1996).

Recently, a pseudodimer model of TCR activation was proposed, providing a theoretical background of how soluble MHC agonist-MHC self-peptide heterodimers can activate T cells (Krogsgaard et al., 2005). Our permissive geometry model provides an alternative explanation. Preformed TCR-CD3 oligomers (Schamel et al., 2005), which bind dimeric MHCp with higher avidity than TCR-CD3 monomers, are in a nonpermissive geometry (Figure 7B). Binding of the MHCp heterodimers to the oligomeric TCR-CD3 would induce the permissive geometry and therefore T cell triggering (Figure 7D). The role of CD4 would be to stabilize the interaction between MHCp and TCR-CD3 and to recruit additional kinases.

Lastly, we show that removal of MHCp tetramers or stimulating antibody reverts the conformational change in the TCR-CD3. Thus, the conformational change is not the molecular event that marks activated TCR-CD3s after MHCp dissociation, which could lead to TCR-CD3 internalization (Coombs et al., 2002), or accumulation during serial triggering (Valitutti et al., 1995). It might be, however, one of the events that directly communicate MHCp binding to the cytoplasmic signaling machinery. Consequently, the duration of TCR-CD3 ligand engagement can be measured intracellularly as proposed by the kinetic proofreading model (McKeithan, 1995). In fact, the combination of an avidity proofreading model with the requirement of conformational changes for T cell activation allowed us to propose recently a thermodynamic model that accounts for the paradox of the high sensitivity and low affinity of the MHCp-TCR-CD3 interaction (Schamel et al., 2006).

In summary, we propose a permissive geometry model that might explain how ligand binding to the rigid TCRαβ subunits is communicated into a conformational change at the cytoplasmic tail of CD3ε, which is necessary, but not sufficient, for full TCR-CD3 triggering and T cell activation.

EXPERIMENTAL PROCEDURES

Cells

We generated the expression vector pSRscTCRβ that encodes a leader peptide, a signal peptidase cleavage site, the NIP-specific

single chain Fv fragment (sc), a linker with the LDGSGGDV sequence, and the mature human Vβ3 HA1.7 chain. The sc was taken from the plasmid pL(-I)VHVL-XhRSI-B (Schamel et al., 2003) and the TCRβ sequence from pJ6β (Hewitt et al., 1992). pSRscTCRβ was transfected into the human Jurkat-derived TCRβ-negative line 31-13 to yield 31-13.scTCRβ (Figure 2A).

The murine T cell line SRD10 and the T1 hybridoma (T1.4 $CD8^-$ and T1 $CD8^+$) have been described (Luescher et al., 1995; Rojo and Janeway, 1988). Murine A20 cells were used as antigen-presenting cells. All cells were maintained in complete RPMI-1640 with 5% serum. T cell clones from T1 transgenic mice were culture as described (Doucey et al., 2003). Human peripheral blood mononuclear cells were isolated from healthy donors according to the local ethics committees on human experimentations via a Ficoll gradient and cotransfected with the pSRscTCRβ and pCMV-GFP plasmids with the Human T Cell Nucleofector Kit (Amaxa GmbH).

Antibodies and Reagents

The rabbit anti-ζ antiserum 448 has been described (San Jose et al., 1998). The following antibodies were used: UCHT1 (anti-human CD3, P. Beverly, UK), 145-2C11 (anti-mouse CD3, J. Bluestone, USA), anti-sc (Ac146, M. Reth, Germany), 3D3 (anti-mouse Vα/β, J.M. Rojo, Spain), and anti-β2microglobulin (T. Dick, Germany). Other antibodies were purchased as follows: OKT3 (anti-human CD3) from Ortho, anti-mouse IgG-PE or IgG-FITC, anti-mouse kappa from Southern Biotech, anti-phospho-LAT (Y191) from Cell Signaling, anti-activated MAPK (12D4) from Nanotools, and anti-phopho-PLCγ (Y383), anti-actin (I-19), and anti-CD3ε (M20) from Southern Biotech. OKT3-Fab fragments were prepared with the Immunopure IgG1-Fab Preparation kit and confirmed by SDS-PAGE and immunoblotting. Secondary antibodies for western blot were obtained from Southern Biotech. NIP (nitro-iodo-phenol)-conjugated BSA (15 haptens per BSA molecule) and free NIP were purchased from Biosearch Technologies (Novato, CA). All NIP peptides were synthesized by IRIS Biotech. Streptavidin-PE was purchased from Molecular Probes. PbCS(ABA) peptide (pepABA) was synthesized as described (Luescher et al., 1991). Soluble monomeric, dimeric, and tetrameric K^dpepABA complexes were prepared as described (Cebecauer et al., 2005; Gregoire et al., 1996; Kalergis et al., 2000).

Cell Stimulations and Lysis

Cells were harvested, resuspended in medium without serum, and incubated 1 hr at 37°C prior to stimulation with the indicated stimulus at 37°C. Alternatively, cells were resuspended in PBS with 2% serum and 0.01% NaN_3 and stimulated at 0°C. The different NIP-coupled reagents were used at the same concentration of the NIP hapten per stimulation. The optimal concentration to stimulate 31-13.scTCRβ cells was determined empirically based on the induced tyrosine-phosphorylation in cellular lysates (data not shown). This concentration (2.8×10^4 NIP molecules/ml) was used in all experiments. T1.4 cells were incubated with the K^dpepABA complexes in medium without serum for 1 hr at 0°C. After UV irradiation, cells were harvested and lysed. A20 cells were loaded overnight with 1 μM of PbCS(ABA) peptide by incubation in complete medium with 1% serum. After PBS washing, A20 and T1 hybridoma cells were brought into close contact by centrifugation and incubated 10 min at 37°C. A ratio of four APCs per T cell was used. Upon UV irradiation and 1% formaldehyde fixation in PBS, cells were lysed. Membrane fractions were prepared by disrupting 3×10^7 cells in hypotonic buffer (10 mM HEPES [pH 7.4], 42 mM KCl, 5 mM $MgCl_2$, and protease inhibitors) with a Dounce homogenizer and pelleting the membranes in an ultracentrifuge at 150,000 × g. NIP elution was performed by incubation with 1 mM of free NIP 1 hr at 4°C. All lyses were done in 1 ml lysis buffer containing 20 mM TrisHCl (pH 8), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 500 μM sodium orthovanadate, 1 mM NaF, and 0.5% Brij96.

SH3.1(Nck) Pull-Down Assay, Immunoprecipitation, and Immunoblotting

Postnuclear fractions were subjected to the SH3.1(Nck) pull-down assay as described (Gil et al., 2002). TCR-CD3 immunoprecipitations were performed with 5 μg of anti-CD3 antibodies overnight at 4°C. Phosphotyrosine immunoprecipitations were performed with PT-66 agarose from Sigma. Samples were subjected to SDS-PAGE separation and transferred to PVDF membranes. Immunoblotting was performed by conventional methods.

T Cell Activation Assays

Ca^{2+} Influx

Cells resuspended in medium with 1% serum were incubated with 5 μg/ml of Indo-1 and 0.5 μg/ml of pluronic F-127 (both Molecular Probes) 45 min at 37°C. After washing, cells were resuspended in medium with 1% serum and kept on ice. Ca^{2+} response was induced by addition of the indicated stimulus 1 min after starting to record the ratio of Ca^{2+}-bound Indo-1 versus unbound Indo-1 with a LSRII fluorescence spectrometer (Becton Dickinson). Data were analyzed with the FloJo 6.1 software.

Luciferase Assay

For the measurement of transcriptional activity, 3×10^7 cells were transiently transfected with 30 μg of NF-AT-luciferase (Hoey et al., 1995) or NF-κB-luciferase (Minguet et al., 2005) reporter plasmids by electroporation. Cells were grown for 24 hr, harvested, resuspended in medium with 0.5% serum, and stimulated 6 hr as indicated. After harvesting, the cells were lysed and luciferase activity measured with the Luciferase assay system from Promega.

Upregulation of CD69

Cells were stimulated for 24 hr with the indicated stimuli. After harvesting, cells were stained with an CD69-PE antibody (Caltag Laboratories) and analyzed by flow cytometry.

Supplemental Data

Six Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/26/1/43/DC1/.

ACKNOWLEDGMENTS

We thank S. Ernst for expert technical assistance, D. Dojcinovic for the pepABA reagents, and A. Spurkland, P. Nielsen, and J. Kirberg for critical reading of the manuscript. We especially thank M. Reth for his scientific support. S.M. was supported by the European Union-founded grant EPI-PEP-VAC, B.A. by grant SAF2006-01391 from the Comision Interministerial de Ciencia y Tecnología, and W.W.A.S by the Deutsche Forschungsgemeinschaft through the Emmy Noether program and the SFB620. The institutional support of the Fundacion Ramon Areces to the Centro de Biologia Molecular Severo Ochoa is acknowledged.

Received: June 16, 2006
Revised: October 10, 2006
Accepted: October 27, 2006
Published online: December 21, 2006

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